

---

## Review

# Potential of a mutant-based reverse genetic approach for functional genomics and molecular breeding in soybean

Toyoaki Anai\*

Laboratory of Plant Genetics and Breeding, Faculty of Agriculture, Saga University, Honjyo-machi 1, Saga 840-8502, Japan

---

Mutant-based reverse genetics offers a powerful way to create novel mutant alleles at a selected locus. This approach makes it possible to directly identify plants that carry a specific modified gene from the nucleotide sequence data. Soybean [*Glycine max* (L.) Merr.] has a highly redundant paleopolyploid genome (approx. 1.1 Gb), which was completely sequenced in 2010. Using reverse genetics to support functional genomics studies designed to predict gene function would accelerate post-genomics research in soybean. Furthermore, the novel mutant alleles created by this approach would be useful genetic resources for improving various traits in soybean. A reverse genetic screening platform in soybean has been developed that combines more than 40,000 mutant lines with a high-throughput method, Targeting Local Lesions IN Genome (TILLING). In this review, the mutant-based reverse genetic approach based on this platform is described, and the likely evolution of this approach in the near future.

**Key Words:** reverse genetics, mutagenesis, TILLING, soybean.

---

## Introduction

Soybean (*Glycine max* (L.) Merr.) is one of the most important leguminous crops. As a legume it has symbiotic nitrogen fixation ability, and is a major global source of proteins and oil for human and animal consumption. The genome sequence of soybean (cv. Williams 82) was completed using a whole-genome shotgun method in 2010 (Schmutz *et al.* 2010). Soybean's paleopolyploid genome, which is approximately 1.1 Gb long, comprises ~46,000 predicted genes, of which nearly 75% are multiple copies. The integrated soybean genome database Soybase (<http://www.soybase.org>) provides public access to the nucleotide sequences and other useful information on soybean (e.g., mapped genes, orthologs and markers). The genome sequence information is useful for traditional (forward genetics) analysis. For example, Watanabe *et al.* (2009, 2011) reported map-based cloning of two genes for maturity quantitative trait loci using a residual heterozygous line.

On the other hand, reverse genetic tools and resources have proven to be indispensable for discovering the biological functions of sequenced genes and for developing novel alleles for specific traits. The soybean genome sequence is an essential tool for accelerating reverse genetic studies. Be-

cause the methods of reverse genetics were developed mainly in studies of *Arabidopsis*, many are based on transgenic technology (Table 1). However, transgenic technology is not a realistic approach for commercial crops such as soybean, because developing and maintaining a huge number of transgenic plants is a time-consuming and labor-intensive process. In contrast, mutant-based reverse genetic methods provide a more practical and potent alternative. There are several major benefits of using a mutant population for reverse genetic analysis: (1) The mutagenesis protocols are well established; (2) A large number of independent mutants can be obtained simultaneously; (3) There is no legal restrictions on the handling and transfer of the mutants, unlike with transgenic plants. Dozens of reverse genetic methods for screening mutant populations have been developed around different mutagens and mutant types. These include the Deletagene (Delete-a-gene) method (Li *et al.* 2001), high-resolution melting (HRM; Gady *et al.* 2009), comparative genomic hybridization (CGH; Bolon *et al.* 2011), and Targeting Local Lesions IN Genomes (TILLING; McCallum *et al.* 2000).

This review provides a brief overview of recent advances in the reverse genetics of crop plants. Furthermore, a mutant collection and TILLING-based high-throughput screening system is described. This paper concludes with the likely future perspectives for the molecular breeding of soybean.

---

Communicated by Duncan Vaughan

Received October 13, 2011. Accepted November 14, 2011.

\*Corresponding author (e-mail: [anai@cc.saga-u.ac.jp](mailto:anai@cc.saga-u.ac.jp))

**Table 1.** Comparison of typical methods used for reverse genetic studies of plants

Method	Resource type	Mutation specificity	Mutation stability	Allelic variations	Screening throughput	Reference
Gene silencing	Recombinant	Uncontrollable	Unstable	Uncontrollable	Depends on the host <sup>‡</sup>	Schwab <i>et al.</i> (2006)
Overexpression	Recombinant	Uncontrollable	Unstable	Uncontrollable	Depends on the host <sup>‡</sup>	Ichikawa <i>et al.</i> (2006)
T-DNA tagging	Recombinant	Random	Stable	Limited	Depends on the host <sup>‡</sup>	Sessions <i>et al.</i> (2002)
Transposon tagging	Recombinant*	Random	Stable	Limited	Depends on the host <sup>‡</sup>	Tissier <i>et al.</i> (1999)
Zinc-finger nuclease	Recombinant	Specific	Stable	Possible	Low	Lloyd <i>et al.</i> (2005)
Homologous recombination	Recombinant	Specific	Stable	Possible	Very low	Shaked <i>et al.</i> (2005)
Deletagene	Non-recombinant	Random	Stable	Limited	High	Li <i>et al.</i> (2001)
TILLING	Non-recombinant	Random	Stable	Available	Medium	McCallum <i>et al.</i> (2000)

\* In the case of rice, a highly active endogenous transposon (*Tos17*) is available for use in the transposon tagging system (Miyao *et al.* 2007).

<sup>‡</sup> Transformation efficiency of the host plant species is the bottleneck for these systems.

### Reverse genetic screening for different types of mutant resource

The size and complexity of a mutant resource is the most important factor for successfully screening mutants by means of the reverse genetic approach. Since the features of a mutant resource depend mainly on the types and doses of mutagens used to create it, the choice of screening method is also important. For example, ionizing radiation (e.g., X-rays, gamma rays and fast neutrons) induces mainly nucleotide deletions of various sizes at a relatively low density (Cecchini *et al.* 1998, Shirley *et al.* 1992). In this case, the large deletions ( $\geq 5$  kb) may completely eliminate multiple functional loci and their functions, whereas the small deletions (1 to 100 b) lead to partial loss of nucleotide sequences, and frequently induce frame-shift, amino acid-truncation, or mis-splicing mutations that lead to complete loss of function of the target genes (Anai *et al.* 2005, 2008, 2012).

In contrast, several popular chemical mutagens [e.g., ethylmethane sulfonate (EMS) and *N*-methyl-*N*-nitrosourea (NMU)] induce mainly single-base substitutions at high density (Greene *et al.* 2003, Satoh *et al.* 2010). The base substitutions induce not only effective mutations such as non-sense, mis-sense, and mis-splicing mutations, but also gene-silencing mutations. These chemical mutagens are attractive because they can take advantage of smaller mutant populations than the other methods. However, the detection of mutants carrying a specific base substitution or small deletion is more difficult than for large deletions.

Two potent screening methods, Deletagene and CGH, are appropriate choices for obtaining mutants with large deletions. The PCR-based Deletagene approach is a simple high-throughput screening method that uses highly pooled (~2500 lines/pool) genomic DNAs prepared from mutant lines treated with fast neutrons as templates (Li *et al.* 2001). In this method, it is necessary to position both primers outside of the deleted region; however, it is difficult to accurately predict the size of each deletion, so designing the primers is a rate-limiting step. The hybridization-based CGH approach is a traditional dot-blot-like method, which uses a high-density microarray containing about 700 000 unique

probes to screen a population irradiated with fast neutrons (Bolon *et al.* 2011). Because of the low mutation rate in these two types of lines, it is necessary to use a large mutant population in both methods. In addition, the cost of a CGH microarray is currently so expensive that it is difficult to use the microarrays to directly screen a specific mutant from a large mutant population.

Currently, the TILLING approach is one of the most attractive screening methods for mutant-based reverse genetics. This method has several advantages over the other methods used to detect single-nucleotide polymorphisms because of its high sensitivity and throughput. TILLING was originally reported as a method to detect mismatches in heteroduplex DNA when denaturing high-performance liquid chromatography was used (McCallum *et al.* 2000). Subsequently, it was modified to use a mismatch-specific nuclease (CEL I) and the LI-COR gel imaging system with fluorescent dye-labeled primers to improve its throughput (Colbert *et al.* 2001). CEL I can efficiently cleave both the base substitutions induced by chemical mutagens and the small deletions induced by ionizing radiation (Oleykowski *et al.* 1998). For this reason, the mutant resources used with the TILLING method have been developed mainly by means of treatment with EMS, but some research groups have successfully isolated several mutants from populations treated with other mutagens (Table 2). These results indicate that the mutant resources obtained from various mutagen treatments could be widely adapted to the TILLING method.

### A TILLING platform for soybean

The TILLING approach has three main advantages for crop species: (1) It can generate a wide range of genetic alterations in addition to loss-of-function alleles, including hypomorphic, hypermorphic and neo-morphic mutations. (2) It can produce permanent and heritable mutations. (3) It can be adapted to polyploid species and functionally redundant genes. Because these benefits are particularly attractive for crop improvement efforts, reverse genetics projects that use the TILLING method are now under way for many crop species, including barley, maize, pea, rice, soybean, tomato and

**Table 2.** Overview of the mutant resources used in the TILLING method in various plant species

Mutagen	Mutation type	Plant species	Mutagen dose	Mutation frequency	Reference
<b>Chemical agents</b>					
EMS	Point mutations	<i>Arabidopsis</i>	20–40 mM	3.3/Mb	Greene <i>et al.</i> (2003)
EMS	Point mutations	Barley	20–30 mM	1.0/Mb	Caldwell <i>et al.</i> (2004)
EMS	Point mutations	<i>Brassica napus</i>	24–48 mM	7.6–24.1/Mb	Wang <i>et al.</i> (2008)
EMS	Point mutations	<i>Brassica rapa</i>	24–32 mM	16.7/Mb	Stephenson <i>et al.</i> (2010)
EMS	Point mutations	Maize	5 mM*	0.9–2.1/Mb	Weil and Morde (2007)
EMS	Point mutations	<i>Medicago</i>	12 mM	2.1/Mb	Le Signor <i>et al.</i> (2009)
EMS	Point mutations	Pea	4 mM	1.5/Mb	Triques <i>et al.</i> (2007)
EMS	Point mutations	Peanut	30–96 mM	0.9–1.1/Mb	Knoll <i>et al.</i> (2011)
EMS	Point mutations	Rice	130 mM	3.8/Mb	Till <i>et al.</i> (2007)
EMS	Point mutations	Sorghum	8–24 mM	1.9/Mb	Xin <i>et al.</i> (2008)
EMS	Point mutations	Soybean	40–50 mM	1.8–7.1/Mb	Cooper <i>et al.</i> (2008)
EMS	Point mutations	Soybean	30–40 mM	0.3–1.3/Mb	Present study
EMS	Point mutations	Tomato	56–80 mM	1.7–3.1/Mb	Minoia <i>et al.</i> (2010)
EMS	Point mutations	Wheat	60–96 mM	41.7/Mb	Slade <i>et al.</i> (2005)
EMS	Point mutations	Wheat (durum)	60–80 mM	25.0/Mb	Slade <i>et al.</i> (2005)
NMU	Point mutations	Rice	1 mM*	7.4/Mb	Suzuki <i>et al.</i> (2008)
NMU	Point mutations	Soybean	2.5 mM	7.1/Mb	Cooper <i>et al.</i> (2008)
NaN <sub>3</sub>	Point mutations	Barley	10 mM	2.6/Mb	Talamè <i>et al.</i> (2008)
<b>Physical agents</b>					
$\gamma$ -rays	Deletions / point mutations	Rice	500 Gy	0.2/Mb	Sato <i>et al.</i> (2006)
X-rays	Deletions	Soybean	200 Gy	0.1/Mb	Present study

EMS, ethylmethane sulfonate; NMU, *N*-methyl-*N*-nitrosourea

\* These mutant populations were developed from progeny pollinated with mutagen-treated pollen.

wheat (Table 2). In these projects, several thousands or tens of thousands of mutant plants are necessary to isolate specific mutants.

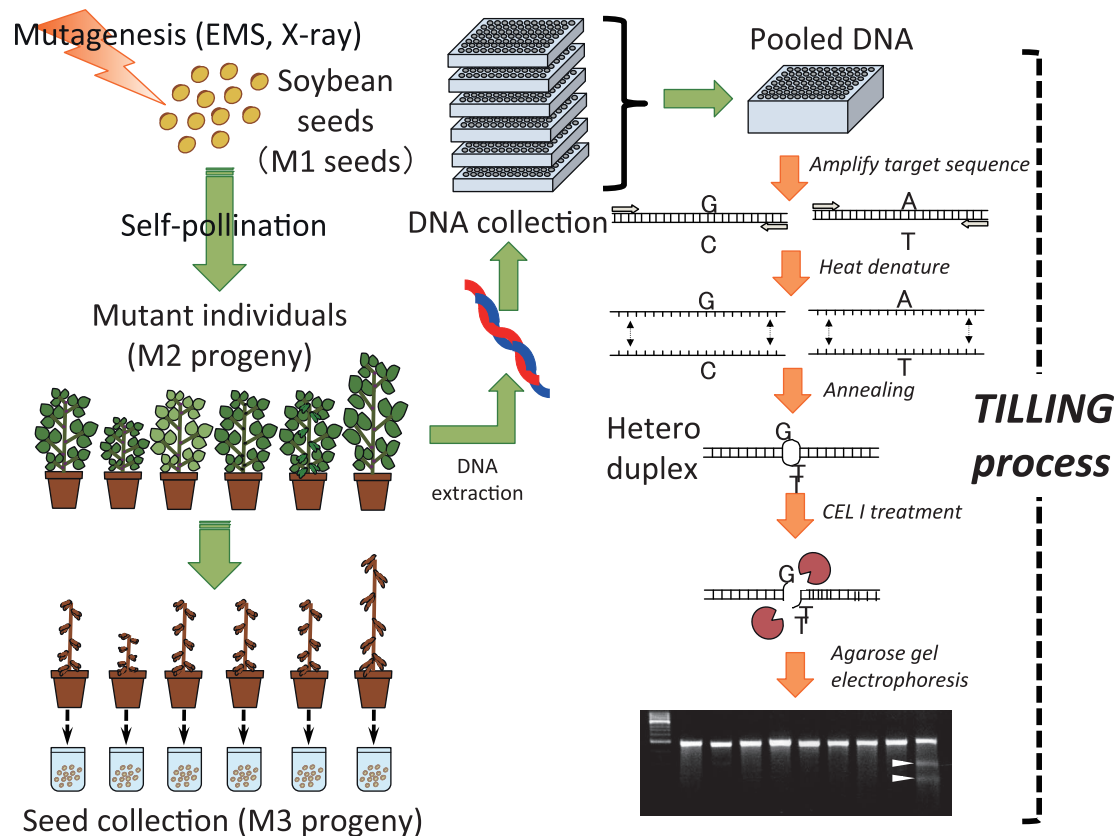
In soybean, Cooper *et al.* (2008) first reported the development of a TILLING system. They developed three EMS-treated populations and an NMU-treated population, and estimated mutation rates ranging from 1.8 to 7.1/Mb. Dierking and Bilyeu (2009) successfully isolated novel mutant alleles for the raffinose synthase gene (*RS2*) and the omega-6 fatty acid desaturase gene (*FAD2-1A*) using this system. We have developed a soybean mutant population consisting of more than 40 000 lines, and have developed an improved TILLING method adapted for high-throughput screening (Fig. 1). This mutant population contains six sub-populations that have mutation rates ranging from 0.1 to 1.3/Mb that were generated through the use of X-rays, EMS, or a combination of the two (Fig. 2). In the TILLING protocol, the cleavage with a mismatch-specific nuclease and the gel electrophoresis are the most important steps. Generally, increasing the mutation density is thought to be a good idea to reduce the size of the population, but a mutant containing too many mutations would be difficult to analyze in terms of its phenotype and difficult to use as breeding material. For these reasons, the mutation rate in the TILLING population must be carefully considered so that it will be appropriate for the purpose of the mutants that are obtained. The activity of the mismatch-specific nuclease has been detected in many plant species (Oleykowski *et al.* 1998). For TILLING, the most commonly used CEL I was obtained from celery, and Yang

*et al.* (2000) reported that CEL I preferentially recognized particular mismatches. Triques *et al.* (2007) reported that recombinant ENDO1, which is a mismatch-specific *Arabidopsis* endonuclease, strongly recognized many types of mismatches and was useful for TILLING. In our system, we detect CEL I-digested double-strand DNA fragments that are pre-stained with a highly sensitive fluorescent dye and separated by means of agarose gel electrophoresis (Fig. 1). We could detect all types of mismatch with this approach. The possible reason of this result may be the presence of two different heteroduplexes originated from both strands of PCR product and/or the higher CEL I concentration optimized for double-strand cleavage system. However, the frequency of false-positive cleavages may be increased by the substrate specificity of CEL I.

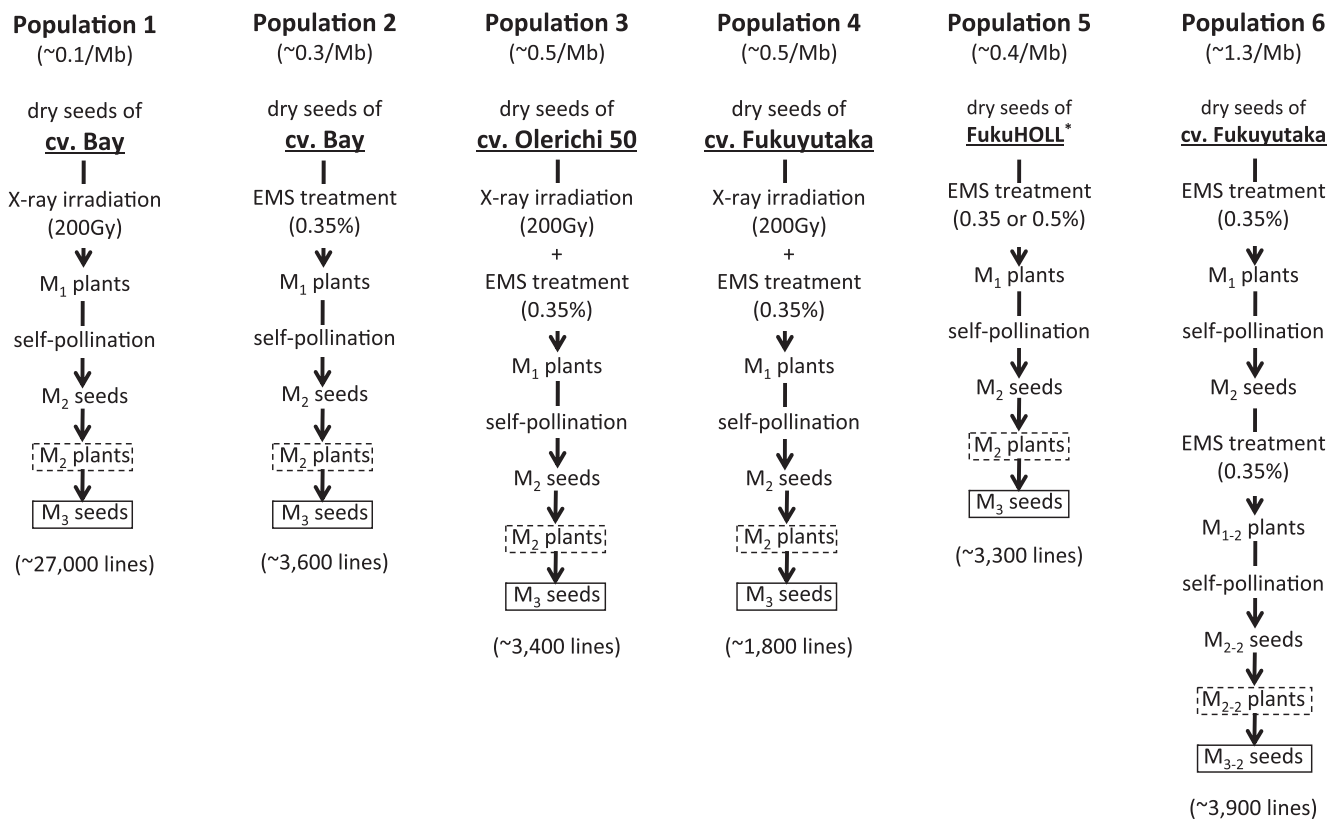
We have also constructed a high-throughput soybean TILLING system that enables a single person to screen about 6000 independent mutant lines in a day. We have successfully isolated some novel alleles of genes that control maturity (Watanabe *et al.* 2009, 2011) and of a fatty acid desaturase gene (Hoshino *et al.* 2010) using this platform.

### Future prospects for reverse genetics in soybean

During the past decade, large-scale genome sequencing projects have been completed in several major crop species, including soybean, and a huge volume of nucleotide sequence data corresponding to individual genes is now available from public databases. Thus, the development of robust



**Fig. 1.** Outline of the development of a soybean mutant population and the process of mutant screening employing the TILLING approach.



**Fig. 2.** Summary of the soybean mutant populations developed at Saga University. \* FukuHOLL is an experimental line that has been described previously (Hoshino *et al.* 2010).

tools for linking the nucleotide sequence of a particular gene to the phenotype produced by its alleles has been desired by the research community. The mutant-based reverse genetic approach described in this paper, which can create novel alleles associated with “perfect markers” (Varshney *et al.* 2006) for a particular gene, has the potential to allow breeders to improve a range of crops without using transgenic technology. This approach can therefore overcome the difficulty of conventional breeding programs, such as the functional redundancy of polyploid genomes and the pyramiding of multiple alleles. This approach may shift the perception of genetic resources in agricultural studies, because it brings about not only loss-of-function mutants with various degrees of impairment but also provides gain-of-function mutants. In fact, a novel mutant virus-resistance allele of the *eIF4E* gene was obtained from EMS-mutagenized tomato populations using the TILLING approach (Piron *et al.* 2010).

To efficiently generate gain-of-function alleles from a mutant population, it is necessary to drastically increase the throughput of TILLING. In recent years, new DNA sequencing technologies that provide very high throughput at a relatively low price, the so-called “next-generation sequencing” (NGS) technologies, have been progressively developed, and the cost of sequencing massive amounts of DNA has rapidly decreased (Ansorge 2009). This progress of NGS will have important implications for achieving next-generation reverse genetics, because the most secure way to identify mutations is by comparison of nucleotide sequences between wild-type and mutant alleles. Most recently, Tsai *et al.* (2011) reported novel TILLING method using NGS, Illumina GA platform, but this protocol contains cumbersome process for separation of mutation signal from large background noise. The current read length of NGS is much shorter than that of Sanger sequencing, so a longer read length (at least 400 to 500 bp/read) will be necessary to adapt NGS to efficient reverse genetic screening. However, Schadt *et al.* (2010) believe that the read-length problem will be overcome within a few years, after which mass-sequencing of multi-dimensionally pooled mutant DNAs will become a major tool for reverse genetic screening. The mutant resources of soybean will become increasingly important for both functional genomics research and developing breeding materials as these tools mature.

## Acknowledgments

This work was partially supported by the Ministry of Agriculture, Forestry and Fisheries of Japan [Genomics for Agricultural Innovation SOY2008] and by a Grant-in-Aid for Scientific Research (C) 22580006 from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## Literature Cited

- Anai, T., T. Yamada, T. Kinoshita, S.M. Rahman and Y. Takagi (2005) Identification of corresponding genes for three low- $\alpha$ -linolenic acid mutants and elucidation of their contribution to fatty acid biosynthesis in soybean seed. *Plant Sci.* 168: 1615–1623.
- Anai, T., T. Yamada, R. Hideshima, T. Kinoshita, S.M. Rahman and Y. Takagi (2008) Two high-oleic-acid soybean mutants, M23 and KK21, have disrupted microsomal omega-6 fatty acid desaturase, encoded by *GmFAD2-1a*. *Breed. Sci.* 58: 447–452.
- Anai, T., T. Hoshino, N. Imai and Y. Takagi (2012) Molecular characterization of two high-palmitic-acid mutant loci induced by X-ray irradiation in soybean. *Breed. Sci.* 61: 631–638.
- Ansorge, W.J. (2009) Next-generation DNA sequencing techniques. *New Biotech.* 25: 195–203.
- Bolon, Y.-T., W.J. Haun, W.W. Xu, D. Grant, M.G. Stacey, R.T. Nelson, D.J. Gerhardt, J.A. Jeddelloh, G. Stacey, G.J. Muehlbauer *et al.* (2011) Phenotypic and genomic analyses of a fast neutron mutant population resource in soybean. *Plant Physiol.* 156: 240–253.
- Caldwell, D.G., N. McCallum, P. Shaw, G.J. Muehlbauer, D.F. Marshall and R. Waugh (2004) A structured mutant population for forward and reverse genetics in barley (*Hordeum vulgare* L.). *Plant J.* 40: 143–150.
- Cecchini, E., B.J. Mulligan, S.N. Covey and J.J. Milner (1998) Characterization of gamma irradiation-induced deletion mutations at a selectable locus in *Arabidopsis*. *Mutat. Res.* 401: 199–206.
- Colbert, T., B.J. Till, R. Tompa, S. Reynolds, M.N. Steine, A.T. Yeung, C.M. McCallum, L. Comai and S. Henikoff (2001) High-throughput screening for induced point mutations. *Plant Physiol.* 126: 480–484.
- Cooper, J.L., B.J. Till, R.G. Laport, M.C. Darlow, J.M. Kleffner, A. Jamaï, T. El-Mellouki, S. Liu, R. Ritchie, N. Nielsen *et al.* (2008) TILLING to detect induced mutations in soybean. *BMC Plant Biol.* 8: 9.
- Dierking, E.C. and K.D. Bilyeu (2009) New sources of soybean seed meal and oil composition traits identified through TILLING. *BMC Plant Biol.* 9: 89.
- Gady, A.L.F., F.W.K. Hermans, M.H.B.J. Van de Wal, E.N. van Loo, R.G.F. Visser and C.W.B. Bachem (2009) Implementation of two high throughput techniques in a novel application: detecting point mutations in large EMS mutated plant populations. *Plant Methods* 5: 13.
- Greene, E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden *et al.* (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. *Genetics* 164: 731–740.
- Hoshino, T., Y. Takagi and T. Anai (2010) Novel *GmFAD2-1b* mutant alleles created by reverse genetics induce marked elevation of oleic acid content in soybean seed in combination with *GmFAD2-1a* mutant alleles. *Breed. Sci.* 60: 419–425.
- Ichikawa, T., M. Nakazawa, M. Kawashima, H. Iizumi, H. Kuroda, Y. Kondou, Y. Tsuchihara, K. Suzuki, A. Ishikawa, M. Seki *et al.* (2006) The FOX hunting system: an alternative gain-of-function gene hunting technique. *Plant J.* 48: 974–985.
- Knoll, J.E., M.L. Ramos, Y. Zeng, C.C. Holbrook, M. Chow, S. Chen, S. Maleki, A. Bhattacharya and P. Ozias-Akins (2011) TILLING for allergen reduction and improvement of quality traits in peanut (*Arachis hypogaea* L.). *BMC Plant Biol.* 11: 81.
- Le Signor, C., V. Savoie, G. Aubert, J. Verdier, M. Nicolas, G. Pagny, F. Moussy, M. Sanchez, D. Baker, J. Clarke *et al.* (2009) Optimizing TILLING populations for reverse genetics in *Medicago truncatula*. *Plant Biotech. J.* 7: 430–441.
- Li, X., Y. Song, K. Century, S. Straight, P. Ronald, X. Dong, M. Lassner and Y. Zhang (2001) A fast neutron deletion mutagenesis-based

- reverse genetics system for plants. *Plant J.* 27: 235–242.
- Lloyd, A., C.L. Plaisier, D. Carroll and G.N. Drews (2005) Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 102: 2232–2237.
- McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff (2000) Targeted screening for induced mutations. *Nature Biotech.* 18: 455–457.
- Minoia, S., A. Petrozza, O. D'Onofrio, F. Piron, G. Mosca, G. Sozio, F. Cellini, A. Bendahmane and F. Carriero (2010) A new mutant genetic resource for tomato crop improvement by TILLING technology. *BMC Res. Note* 3: 69.
- Miyao, A., Y. Iwasaki, H. Kitano, J. Itoh, M. Maekawa, K. Murata, O. Yatou, Y. Nagato and H. Hirochika (2007) A large-scale collection of phenotypic data describing an insertional mutant population to facilitate functional analysis of rice genes. *Plant Mol. Biol.* 63: 625–635.
- Oleykowski, C.A., C.R. Bronson Mullins, A.K. Godwin and A.T. Yeung (1998) Mutation detection using a novel plant endonuclease. *Nucleic Acids Res.* 26: 4597–4602.
- Piron, F., M. Nicolai, S. Minoia, E. Piednoir, A. Moretti, A. Salgues, D. Zamir, C. Caranta and A. Bendahmane (2010) An induced mutation in tomato eIF4E leads to immunity to two Potyviruses. *PLoS ONE* 5: e11313.
- Sato, Y., K. Shirasawa, Y. Takahashi, M. Nishimura and T. Nishio (2006) Mutant selection from progeny of gamma-ray-irradiated rice by DNA heteroduplex cleavage using *Brassica* petiole extract. *Breed. Sci.* 56: 179–183.
- Satoh, H., H. Matsusaka and T. Kumamaru (2010) Use of *N*-methyl-*N*-nitrosourea treatment of fertilized egg cells for saturation mutagenesis of rice. *Breed. Sci.* 60: 475–485.
- Schadt, E.E., S. Turner and A. Kasarskis (2010) A window into third-generation sequencing. *Hum. Mol. Genet.* 19: R227–R240.
- Schmutz, J., S.B. Cannon, J. Schlueter, J. Ma, T. Mitros, W. Nelson, D.L. Hyten, Q. Song, J. Thelen, J. Cheng *et al.* (2010) Genome sequence of the palaeopolyploid soybean. *Nature* 463: 178–183.
- Schwab, R., S. Ossowski, M. Riester, N. Warthmann and D. Weigel (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. *Plant Cell* 18: 1121–1133.
- Sessions, A., E. Burke, G. Presting, G. Aux, J. McElver, D. Patton, B. Dietrich, P. Ho, J. Bacwaden, C. Ko *et al.* (2002) A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell* 14: 2985–2994.
- Shaked, H., C. Melamed-Bessudo and A.A. Levy (2005) High-frequency gene targeting in *Arabidopsis* plants expressing the yeast RAD54 gene. *Proc. Natl. Acad. Sci. USA* 102: 2232–2237.
- Shirley, B.W., S. Hanley and H.M. Goodman (1992) Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis* *transparent testa* mutations. *Plant Cell* 4: 333–347.
- Slade, A.J., S.I. Fuerstenberg, D. Loeffler, M.N. Steine and D. Facciotti (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. *Nature Biotech.* 23: 75–81.
- Stephenson, P., D. Baker, T. Girin, A. Perez, S. Amoah, G.J. King and L. Østergaard (2010) A rich TILLING resource for studying gene function in *Brassica rapa*. *BMC Plant Biol.* 10: 62.
- Suzuki, T., M. Eiguchi, T. Kumamaru, H. Satoh, H. Matsusaka, K. Moriguchi, Y. Nagato and N. Kurata (2008) MNU-induced mutant pools and high performance TILLING enable finding of any gene mutation in rice. *Mol. Genet. Genom.* 279: 213–223.
- Talamè, V., R. Bovina, M.C. Sanguineti, R. Tuberosa, U. Lundqvist and S. Salvi (2008) TILLMore, a resource for the discovery of chemically induced mutants in barley. *Plant Biotech. J.* 6: 477–485.
- Till, B.J., J. Cooper, T.H. Tai, P. Colowit, E.A. Greene, S. Henikoff and L. Comai (2007) Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biol.* 7: 19.
- Tissier, A.F., S. Marillonnet, V. Klimyuk, K. Patel, M.A. Torres, G. Murphy and J.D.G. Jones (1999) Multiple independent defective suppressor-mutator transposon insertions in *Arabidopsis*: a tool for functional genomics. *Plant Cell* 11: 1841–1852.
- Triques, K., B. Sturbois, S. Gallais, M. Dalmis, S. Chauvin, C. Clepet, S. Aubourg, C. Rameau, M. Caboche and A. Bendahmane (2007) Characterization of *Arabidopsis thaliana* mismatch specific endonucleases: application to mutation discovery by TILLING in pea. *Plant J.* 51: 1116–1125.
- Tsai, H., T. Howell, R. Nitcher, V. Missirian, B. Watson, K.J. Ngo, M. Lieberman, J. Fass, C. Uauy, R.K. Tran *et al.* (2011) Discovery of rare mutations in populations: TILLING by sequencing. *Plant Physiol.* 156: 1257–1268.
- Varshney, R.K., A. Graner and M.E. Sorrells (2006) Genomics-assisted breeding for crop improvement. *Trends Plant Sci.* 10: 621–630.
- Wang, N., Y. Wang, F. Tian, G.J. King, C. Zhang, Y. Long, L. Shi and J. Meng (2008) A functional genomics resource for *Brassica napus*: development of an EMS mutagenized population and discovery of FAE1 point mutations by TILLING. *New Phytol.* 180: 751–765.
- Watanabe, S., R. Hideshima, Z. Xia, Y. Tsubokura, S. Sato, Y. Nakamoto, N. Yamanaka, R. Takahashi, M. Ishimoto, T. Anai *et al.* (2009) Map-based cloning of the gene associated with the soybean maturity locus *E3*. *Genetics* 182: 1251–1262.
- Watanabe, S., Z. Xia, R. Hideshima, Y. Tsubokura, S. Sato, N. Yamanaka, R. Takahashi, T. Anai, S. Tabata, K. Kitamura *et al.* (2011) A map-based cloning strategy employing a residual heterozygous line reveals that the *GIGANTEA* gene is involved in soybean maturity and flowering. *Genetics* 188: 395–407.
- Weil, C.F. and R.A. Monde (2007) Getting the point-mutations in maize. *Crop Sci.* 47: S60–S67.
- Xin, Z., M.L. Wang, N.A. Barkley, G. Burow, C. Franks, G. Pederson and J. Burke (2008) Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biol.* 8: 103.
- Yang, B., X. Wen, N.S. Kodali, C.A. Oleykowski, C.G. Miller, J. Kulinski, D. Besack, J.A. Yeung, D. Koealski and A.T. Yeung (2000) Purification, cloning, and characterization of the CEL I nuclease. *Biochemistry* 39: 3533–3541.